

Sequence-Specific Cleavage of Huntington mRNA by Catalytic DNA

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The selective loss of neurons in Huntington's disease (HD) is caused by the abnormal expansion of the CAG triplet (>36 repeats) of the HD gene. Although the molecular events that lead to neuronal death are not clear, it is most likely that mutant HD protein operates through a "gain-of-function" mechanism. One possible therapeutic approach that does not require definition of the toxic mechanism(s) involves reduction in the levels of mutant HD protein by decreasing the quantity of translatable HD mRNA. In this report, we demonstrate the first effective destruction of the HD mRNA, using a catalytic DNA—an oligodeoxynucleotide with RNA-cleaving enzymatic activity. We show that the cleavage of HD mRNA by the catalytic DNA occurs in a sequence-specific manner, and leads to significant reduction of HD protein expression in mammalian cells. The catalytic DNAs we have developed are a valuable research tool for studying HD, and may have the therapeutic potential of reducing cellular toxicity caused by mutant HD protein.

Yen L, Strittmatter SM, Kalb RG. Sequence-specific cleavage of huntingtin mRNA by catalytic DNA. Ann Neurol 1999;46:366–373

Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder that causes progressive involuntary choreiform movements, cognitive deterioration,¹ and severe neuronal loss, particularly in the striatum.² The disease is caused by the expansion of a CAG triplet repeat greater than 36 in exon 1 of the HD gene,^{3,4} although the mechanism by which the mutant gene causes neurodegeneration is not clear.^{5–8} Transgenic mice that express an extra copy of mutant human HD gene develop HD-like neurological symptoms,^{9,10} whereas heterozygous knockout mice with an approximate 50% reduction of wild-type HD expression are phenotypically normal.^{11,12} These findings support the view that the disease is caused by a toxic "gain of function" rather than a simple loss of activity. These observations suggest that reducing the level of toxic mutant protein by targeted destruction of the mutant mRNA might slow the pathological process or processes.

Our mRNA targeting strategy depends on the Watson-Crick base pairing between a short catalytic DNA sequence ("DNA enzyme") and an accessible single-stranded region in the HD mRNA. The DNA enzyme has a catalytic core of 15 bases, which performs the endonuclease function, flanked by two substrate-binding arms that can be specified to bind to a particular RNA sequence (see Fig 2).¹³ Unlike antisense oligodeoxynucleotides that may inactivate their target by translation inhibition or other mechanism(s),¹⁴ DNA

enzymes function as a true enzyme, capable of multi-turnover cleavage of target RNAs. Their small size (~30 bases) and high catalytic efficiency are comparable with ribozymes, and the inherent stability of DNA make preparation and use straightforward.^{13,15}

Materials and Methods

Design of DNA Enzymes

The mFold program of Zuker¹⁶ (<http://mfold.wustl.edu/~folder/rna/form1.cgi>) is used to analyze the HD mRNA sequences to identify several potential minimum energy secondary structures. Candidate sites for DNA enzyme cleavage were chosen by analyzing the consensus structures between the possible multiple alternative foldings.

To design DNA enzymes targeting specific sites, we followed the guidelines published by Santoro and Joyce.¹³ The cleavage locations were chosen to occur between an unpaired purine and a paired pyrimidine within putative single-stranded regions. The length of the substrate-binding arms of individual DNA enzymes was determined by calculating the ΔG to be smaller than -24 kCal/mol when hybridized to their target mRNA.^{13,15,17} This corresponds to a length of 16 bases for HD-9, and 21 bases for HD-421. Synthetic oligo DNAs with or without 3'-3' inverted dT were prepared by automated chemical synthesis at Keck Biotechnology Resource Laboratory, Yale University.

In Vitro DNA Enzyme Cleavage

In vitro cleavage was performed by combining DNA enzymes with radiolabeled HD mRNA, and the cleaved prod-

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Received Dec 22, 1998, and in revised form Apr 8, 1999. Accepted for publication Apr 8, 1999.

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ucts were separated by electrophoresis. Radiolabeled HD mRNA containing exons 1 to 6 with 16, 43, or 84 CAG repeats were generated by in vitro transcription in the presence of ^{33}P -UTP (ICN, Irvine, CA), and gel-purified. The in vitro cleavage reaction was performed by incubating radiolabeled HD mRNA with various DNA enzymes (1 μM) in 10 mM Mg $^{2+}$, 100 mM KCl, 50 mM Tris-HCl, 100 mM NH $_4$ Cl, pH 7.3, at 37°C for 35 minutes. Products of reaction were separated by electrophoresis on a 5% polyacrylamide/8 M urea gel and imaged by autoradiography.

The HD expression plasmids used to generate labeled HD mRNA are kindly provided by Dr Christopher Ross, and were sequenced before use. All sequences conformed to previously published results³ with the exception of nine extra nucleotides at the end of CCG repeats, downstream from the CAG repeats in the plasmid containing 16 CAG repeats. This insertion occurred in an inaccessible region of the HD mRNA and, thus, has no impact on our results except to render a slightly longer cleavage fragment.

Determination of Locations of Cleavage within the HD mRNA

To identify the nucleotide at which the DNA enzyme cleaves the HD mRNA, products of the DNA enzyme cleavage were electrophoresed alongside an RNA sequencing ladder. The ladder was generated by partially digesting radiolabeled HD mRNA with RNase T1 (which cuts after each single-stranded guanosine).¹⁸ Short HD mRNAs, covering a region from translational initiation codon AUG to a position 80 nucleotides downstream of the CAG repeats, were 3'-end-labeled with [^{32}P]pCp (ICN) by T4 RNA ligase, and gel-purified. The labeled HD mRNAs were then partially digested with diluted RNase T1 (Boehringer, Indianapolis, IN) under mild denaturing conditions. The actual dilution of RNase T1 used for digestion was determined empirically and a typical range was from 1:100 to 1:500. Conditions for RNase T1 digestion were as follows: 20 mM sodium citrate, 1 mM EDTA, 0.2 $\mu\text{g}/\mu\text{l}$ tRNA, 5.5 M urea, for 5 minutes at 50°C. Products of the DNA enzyme cleavage and the RNA ladder were separated by electrophoresis on a 10% polyacrylamide/8 M urea gel and imaged by autoradiography.

Cotransfection Assay and Immunoblot Analysis

Human embryonic kidney cells (HEK-293) at 50 to 60% confluence were cotransfected with 0.25 μg of HD expression plasmid containing exons 1 to 6 under the control of a cytomegalovirus (CMV) promoter, and with 10 μg of protected DNA enzyme in each 35-mm dish. Transfection was performed by using LipofectAMINE (GIBCO, Grand Island, NY) in 1 ml of serum-free OptiMEM medium (GIBCO), and cells were incubated for 6 hours, followed by the addition of 2 ml of normal medium for another 18 hours. The transfecting medium was then replaced by normal medium containing 1 μM DNA enzymes for another 24 hours. Cell lysates were prepared for western blot analysis 48 hours after initial transfection. Equal amount of protein samples were separated by electrophoresis on a 10% sodium dodecyl sulfate polyacrylamide gel, and blotted with polyclonal antibody AP-194 against the first 17 amino acids of HD protein¹⁹ (kindly provided by Dr Alan Sharp). The mem-

brane was then incubated with a horseradish peroxidase-conjugated anti-rabbit IgG antibody (Sigma, St. Louis, MO), and the bands were detected using the enhanced chemiluminescence (ECL) system. The membrane was then stripped with 2% sodium dodecyl sulfate in 50 mM Tris buffer (pH 7.6) for 4 hours at 60°C, and reprobed with an anti-actin antibody (Sigma). All experiments were performed at least in triplicate. Lane-to-lane variation in the amount of loaded proteins was controlled internally by normalizing the level of HD protein to that of actin.

Results

Our approach for using DNA enzymes to destroy the HD mRNA involves three steps. First, we used the RNA folding program mFold of Zuker^{16,20} to search for potential single-stranded regions within the HD mRNA from exons 1 to 6. On the basis of these predictions, we selected the 15 most probable sites. Second, we designed DNA enzymes that should bind in a sequence-specific manner to each of the selected sites. Third, the cleavage activity was measured by incubating individual DNA enzymes with radiolabeled HD mRNA containing either 16 CAG repeats (wild type), or 43 or 84 CAG repeats (mutants).

The 15 most probable sites, as predicted by the mFold program, and their locations within human HD exons 1 to 6 are indicated in Figure 1a. Each site is named by its position downstream from the 3' end of CAG repeats. Examples of the putative secondary structures close to the CAG repeats and a region approximately 400 bases downstream to the CAG repeats are shown in Figure 1b and c with labeled candidate sites. Of the 15 sites tested in the HD mRNA, DNA enzymes targeted to sites 9 and 421 led to significant cleavage of HD mRNA. The remaining sites either had low cleavage (positions 6, 32, 236, and -29), or resulted in no cleavage at all (positions 0, 3, 41, 50, 152, 370, 539, 543, and 550). A DNA enzyme designed to target the CAG repeats produced only minimal cleavage.

An example of the sequence of the DNA enzyme targeting CAG repeats and at position 0 (referred to as HD-CAG and HD-0 enzyme, correspondingly) are illustrated in Figure 2a. These DNA enzymes contain one catalytic core and two substrate-binding arms designed to bind in a sequence-specific manner to each of the selected sites. The inefficient cleavage by these DNA enzymes of HD mRNA are shown in Figure 2b. The DNA enzyme targeting CAG repeats produced only minimal cleavage (lanes 1–3), with a slight preference for the mutant versus wild-type HD mRNA (compare lane 3 with lane 1). The DNA enzyme targeting at position 0, with one of its arms binding to the CAG repeats and the other arm to the downstream sequence, resulted in virtually no cleavage (lanes 4–6).

The sequences of the two most favorable sites and their corresponding DNA enzymes (referred to as

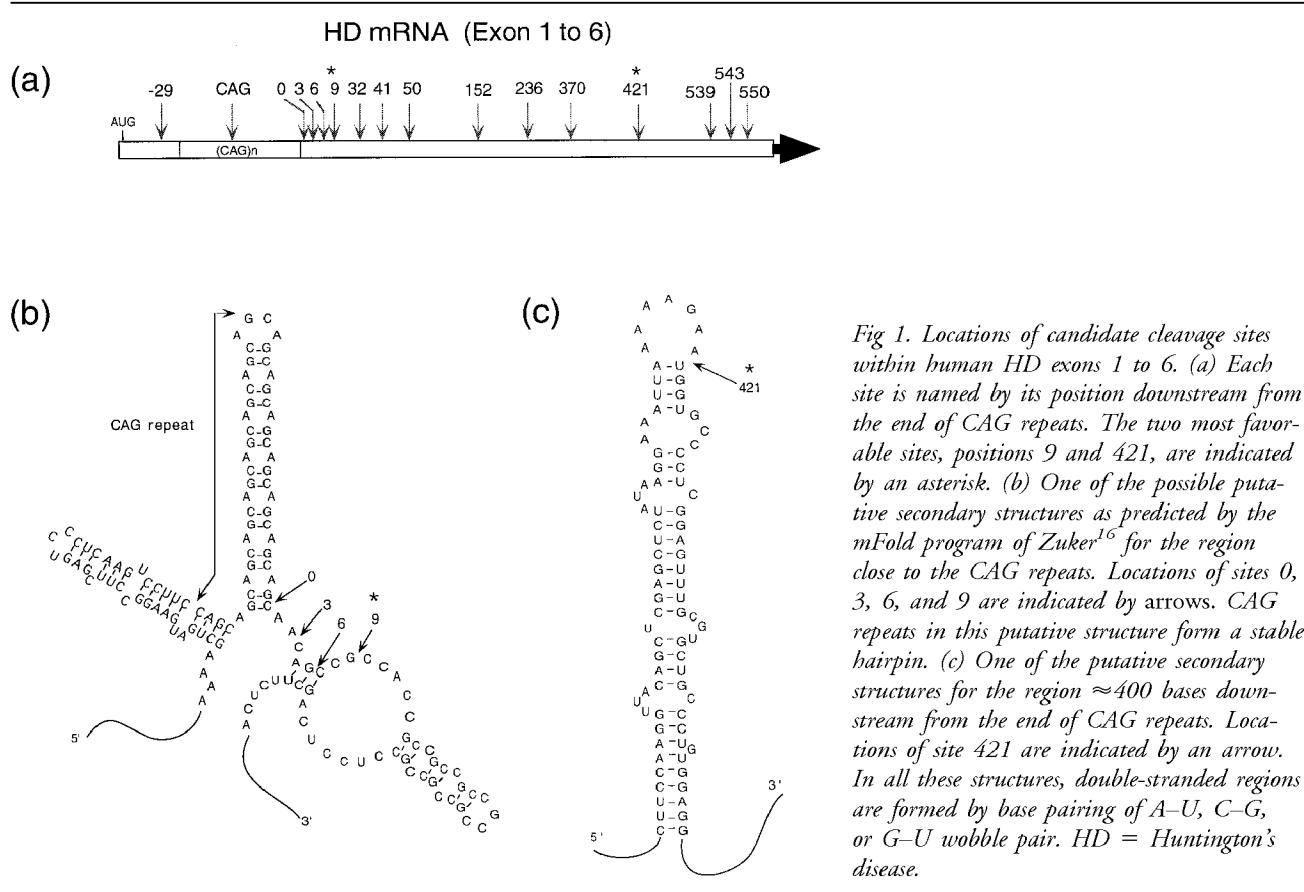


Fig 1. Locations of candidate cleavage sites within human HD exons 1 to 6. (a) Each site is named by its position downstream from the end of CAG repeats. The two most favorable sites, positions 9 and 421, are indicated by an asterisk. (b) One of the possible putative secondary structures as predicted by the mFold program of Zuker¹⁶ for the region close to the CAG repeats. Locations of sites 0, 3, 6, and 9 are indicated by arrows. CAG repeats in this putative structure form a stable hairpin. (c) One of the putative secondary structures for the region ≈400 bases downstream from the end of CAG repeats. Locations of site 421 are indicated by an arrow. In all these structures, double-stranded regions are formed by base pairing of A-U, C-G, or G-U wobble pair. HD = Huntington's disease.

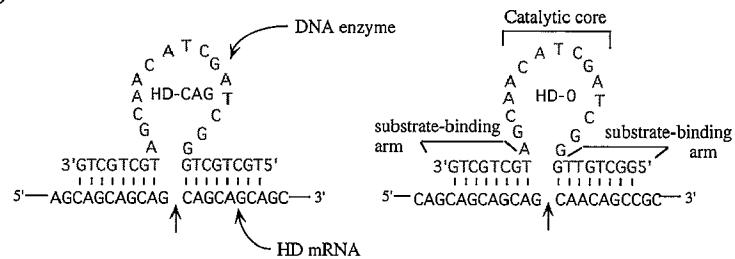
HD-9 and HD-421 DNA enzymes) are illustrated in Figure 3a. Both DNA enzymes cleave the HD mRNA to produce two fragments of the predicted sizes (see Fig 3b, lanes 1–6), suggesting that these DNA enzymes act in a sequence-specific manner. This is further supported by the demonstration that the DNA enzyme with two mismatched nucleotides in the substrate-binding arm exhibited no cleavage activity (see Fig 3b, lanes 7–9; changing the left arm sequence of HD-9 to 3'-GTCGTCGT-5', mutation underlined).

The precise locations of cleavage were determined by the size of liberated 3' fragments on a polyacrylamide gel along with a sequence ladder. This sequence ladder was generated by partial digestion of mildly denatured HD mRNA by RNase T1, an endonuclease that cleaves at single-stranded G.¹⁸ As shown in Figure 4, the liberated 3' fragment migrates to a position approximately 9 bases 3' of the CAG repeats. This indicates that cleavage by the HD-9 enzyme occurred at the expected location in HD mRNA. Cleavage was found to occur at the same site in mRNA containing 84 (see Fig 4a), 43, or 16 CAG repeats (data not shown), indicating that sequence-specific cleavage is unaffected by the number of CAG repeats. These results, together with the findings shown in Figure 3b,

confirm that the DNA enzymes we designed to cleave the HD mRNA in a sequence-specific manner.

Inspection of the partial RNase T1 digestion of HD mRNA reveals relatively weak digestion within the CAG repeats (see Fig 4a, light bands, lane 2), suggesting that stable secondary structures were formed within the CAG repeats. In contrast, the relatively strong digestion (dark bands) by RNase T1 in the region immediately downstream to the CAG repeats indicates that this region is single stranded (as marked by the double asterisks in lane 2). This confirms that the site targeted by HD-9 is indeed located in an accessible, single-stranded region. It also indicates that other factors in addition to "accessibility" are involved in determining a favorable site, since of the four DNA enzymes targeting this region (at positions 0, 3, 6, and 9), only HD-9 results in significant cleavage. Finally, the pattern of partial RNase T1 digestion appeared identical regardless of the number of CAG repeats, suggesting that the expansion of CAG repeats does not alter the secondary structures in the immediate downstream region. Based on these patterns of RNase T1 digestions, a model of the mRNA secondary structure near the CAG repeats is proposed in Figure 4b. In this model, the major part of the CAG repeats forms a

(a)



(b)

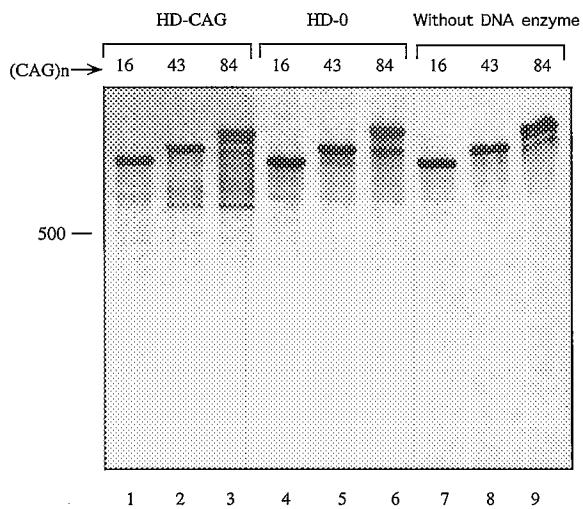


Fig 2. Poor cleavage activity of DNA enzymes targeted to CAG repeats and position 0. (a) Illustrations of the two DNA enzymes (HD-CAG and HD-0) hybridized to their specific target sequences within the HD mRNA. The DNA enzyme (upper strand) contains one catalytic core and two substrate-binding arms. The site of cleavage in the HD mRNA (lower strand) occurs preferentially between an unpaired purine and a paired pyrimidine as indicated by the small vertical arrow. (b) Inefficient cleavage of HD mRNA by both DNA enzymes. DNA enzymes at concentration of 1 μ M are incubated with HD mRNA containing either 16 CAG repeats (lanes 1, 4, and 7), 43 repeats (lanes 2, 5, and 8), or 84 repeats (lanes 3, 6, and 9). The uppermost band of each lane is the uncleaved mRNA, and the lower bands are the cleavage products. Note that the DNA enzyme targeting CAG repeats produced minimal but slightly preferential cleavage for the mutant versus wild-type HD mRNA (lanes 1–3). The DNA enzyme HD-0, with one of its arms binding to the CAG repeats and the other arm to the downstream sequence, resulted in no cleavage (lanes 4–6). In lanes 7 through 9, no DNA was added and these lanes served as the controls. The lower bands seen in lanes 4 through 9 are the products of nonspecific degradation of mRNA, and were not the result of DNA enzyme cleavage. HD = Huntington's disease.

stable hairpin structure regardless of the repeat number, followed by a single-stranded region immediate downstream.

Because the two favorable sites, positions 9 and 421, are separated by several hundred bases, it suggests that binding of one enzyme at the first site will not impede binding at the second site. To test this idea, we combined the two DNA enzymes (each with half of the original concentration) in a single reaction and examined the resultant cleavage products. As shown in Figure 3b, combining the HD-9 and HD-421 DNA enzymes resulted in a higher level of cleavage (~90% in lanes 10–12, vs 71–74% in lanes 1–6), indicating an additive effect. This would be predicted if, by combining two different DNA enzymes, the number of possible favorable sites is doubled although the total concentration of DNA enzymes is unaltered.

To determine if the DNA enzymes can operate in cells to reduce the level of HD proteins, we performed cotransfection assays of various DNA enzymes together with an HD expression plasmid. Because single-stranded DNAs are subjected to degradation in cells, we modified our DNA enzymes by adding a 3'-3' inverted dT to the 3' end.¹⁵ This maneuver increases the

stability of small DNA molecules in cells. After cotransfection into the HEK-293 cells, we assayed for the HD protein expression by immunoblot. As shown in Figure 5, both HD-9 and HD-421 reduced the level of transfected mutant HD protein by at least 85% compared with the controls (see Fig 5, lanes 1 and 2 vs lanes 3 and 4). This level of reduction of HD protein was reproducible and was observed in more than three independent transfection experiments. We used two controls: first, a DNA enzyme that produced no cleavage in vitro (lane 4, HD-0; also see Fig 2, lanes 4–6); and, second, a DNA enzyme identical to HD-421 with the exception of a single base change in the catalytic core that inactivates its endonuclease activity (lane 3, changing the core sequence to 5'-GGCTAGCTGCA ACCA-3', mutation underlined). When compared with the active DNA enzymes, no alteration in HD expression was produced by either of the control DNA enzymes. These results demonstrate that within the cellular milieu, the DNA enzymes were able to reduce the expression of the HD protein significantly, and this reduction is not simply the result of the antisense effect, since a point mutation in the catalytic core with no change in the substrate-binding arms prevented the re-

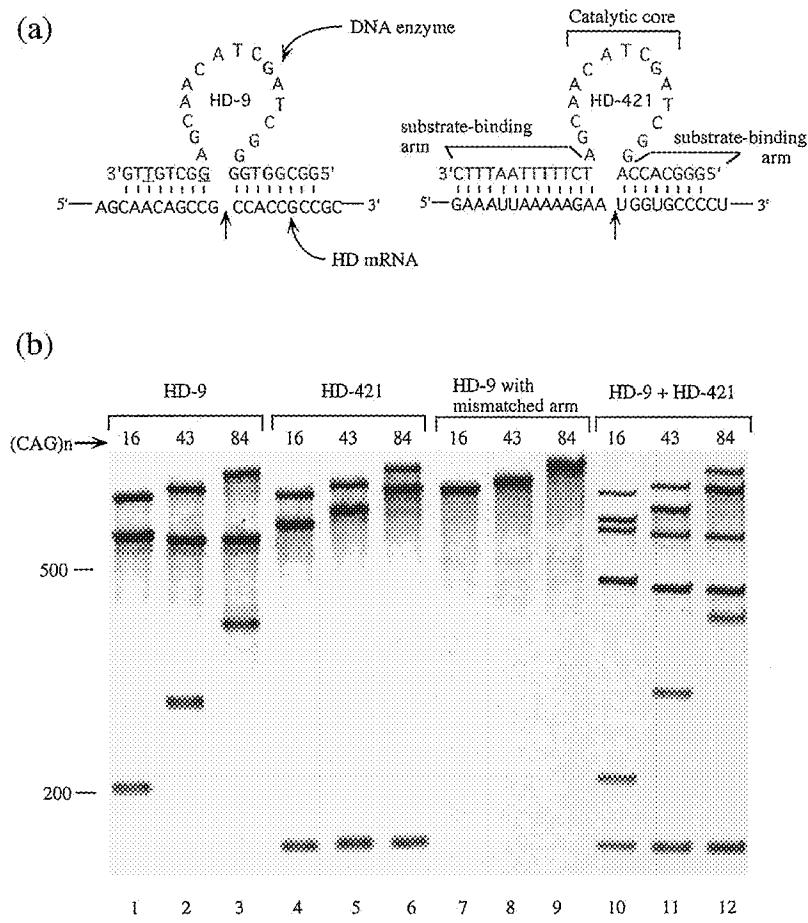


Fig 3. Effective destruction of the HD mRNA at the two most favorable sites. (a) The sequences of the two most favorable sites, positions 9 and 421, and their corresponding DNA enzymes are illustrated. (b) Destruction of HD mRNA by DNA enzymes. DNA enzymes are incubated with HD mRNA containing either 16 CAG repeats (lanes 1, 4, 7, and 10), 43 repeats (lanes 2, 5, 8, and 11), or 84 repeats (lanes 3, 6, 9, and 12). The uppermost band of each lane is the uncleaved mRNA, and the lower bands are the cleavage products. Both DNA enzymes cleaved the HD mRNA to produce two fragments of the predicted sizes (lanes 1–6). In lanes 7 through 9, the underlined “T” and “G” in the arm of HD-9 were substituted by “C” and “T,” and this resulted in no cleavage. In lanes 10 through 12, HD-9 and HD-421 were combined to cleave HD mRNA and this resulted in six fragments of the predicted sizes. The total concentration of DNA enzymes in all lanes is fixed at 1 μ M. Because of a 9-nucleotides insertion found only in plasmid used to generate HD mRNA containing 16 CAG repeats, one of the cleavage fragments was slightly longer than the comparable fragment in neighboring lanes. HD = Huntington's disease.

duction in the level of HD protein. No change in the abundance of endogenous protein actin was observed after transfection with DNA enzymes or with mock transfection.

Discussion

In this study, we custom-designed 15 DNA enzymes, each targeting different sites within putative single-stranded regions of the HD mRNA. Of the 15 tested, DNA enzymes targeted to positions 9 and 421 led to significant cleavage of HD mRNA. We demonstrated that both DNA enzymes operate in a sequence-specific manner to cleave the HD mRNAs, and the cleavage occurred at the predicted locations. When transfected into cells, both DNA enzymes reduced the expression of the HD protein significantly, and this reduction was not the result of the antisense effect. The efficiency of these DNA enzymes to reduce HD proteins in cells suggest that this mRNA-targeting approach may be a useful investigative tool in animal models of HD, and perhaps even have clinical utility.

Based on the patterns of RNase T1 digestions, we proposed a model of the HD mRNA structure (see Fig 4b) in which the major part of the CAG repeats forms

a stable hairpin structure, and the hairpin is followed by a single-stranded region. This model differs from the computer-predicted structure in Figure 1b in that a small portion of the 3' end of the CAG repeats is actually single stranded. Because the pattern of RNase T1 digestions appeared identical regardless of the number of CAG repeats, it suggests that the expansion of CAG repeats simply produced a longer hairpin without altering the secondary structures in the immediate downstream region. This may explain why the DNA enzyme HD-9, which targets the single-stranded region immediately downstream to the CAG repeats, exhibited no preferential cleavage of the mutant versus wild-type HD mRNA.

In fact, none of the DNA enzymes we designed led to preferential cleavage of the mutant versus wild-type HD mRNA. Although slightly preferential cleavage of the mutant versus wild-type HD mRNA was seen with a DNA enzyme targeting the CAG repeats directly, the inefficiency of its cleavage precludes its use. Even if its efficiency could be optimized, such a DNA enzyme would not be specific to HD mRNA, because at least eight other genes are known to contain CAG repeats.^{21,22} The DNA enzyme targeting position 0

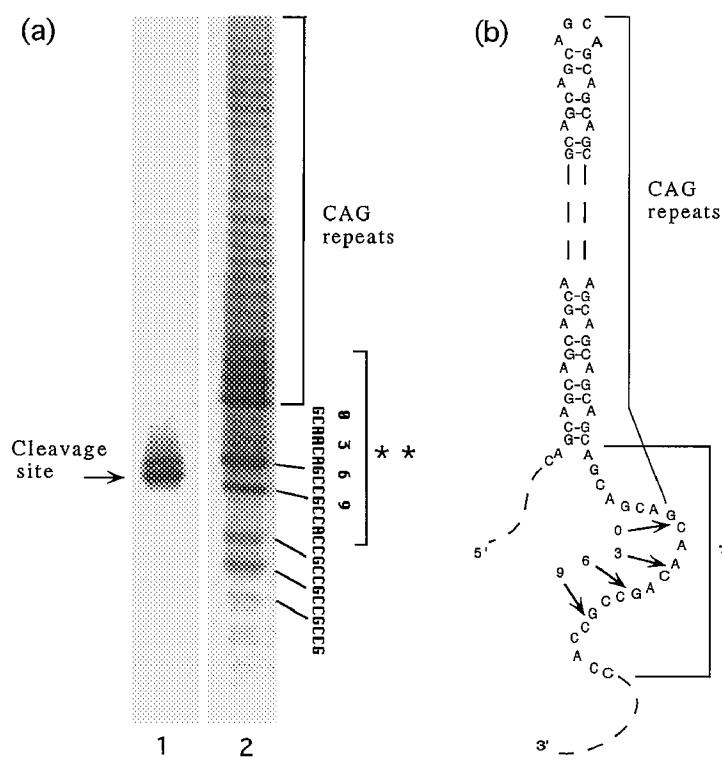


Fig 4. Cleavage occurs at the predicted locations within HD mRNA. (a) In lane 1, a short HD mRNA containing 84 CAG repeats was 3'-end-labeled with [32 P]pCp, and digested by HD-9. The ladder at lane 2 was produced by partial digestion of the same labeled HD mRNA with RNase T1 under mild denaturing conditions. RNase T1 cleaves only at G in single-stranded regions, and therefore generates a sequence ladder. The cleaved 3' fragment by HD-9 migrates to a position approximately 9 bases 3' of the CAG repeats as indicated by an arrow in lane 1. A minor cleavage product is also seen 1 base upstream, suggesting a degree of flexibility of the catalytic core in attacking phosphodiester linkages within the targeted locations. Locations of candidate sites 0, 3, 6, and 9 are labeled on the right side of the sequence. Possible single-stranded region is marked by the double asterisks in lane 2. (b) A model of the mRNA secondary structure near the CAG repeats based on the results of RNase T1 digestions. The CAG repeats form a stable hairpin structure, whereas the immediate downstream region is single stranded. Note that a small portion of the CAG repeats at the 3' end is part of the single-stranded region. Locations of candidate sites 0, 3, 6, and 9 are labeled on the sequence, and the single-stranded region is marked by the double asterisks. HD = Huntington's disease.

(HD-0), with one of its arms binding to the CAG repeats and the other arm binding to the HD-specific sequence, could theoretically provide the required specificity to HD mRNA. Unfortunately, as shown in Figure 2, such a DNA enzyme resulted in no cleavage. Specific targeting of mutant HD thus may represent an unusual challenge, since the difference between mutant and wild-type HD sequence lies in the number of CAG repeats, rather than the addition of unique sequences.

Although our optimal DNA enzymes do not discriminate between mutant and wild-type HD mRNA, an intriguing possibility is that a reduction in the level of mutant HD by DNA enzymes may decrease pathological events, whereas the accompanying reduction in the wild-type HD may be phenotypically neutral. It is known that huntingtin plays a critical role in early embryonic development, because homozygous targeted disruption of the murine HD gene is embryonic lethal,^{11,12,23} and a greater than 50% reduction of HD expression leads to perinatal lethality.²⁴ Whether huntingtin is required for normal function in adults is unclear and would require experiments using conditional knockout animals. Several studies suggest that if the HD protein is needed for normal function in adults, its role might be fulfilled by 50% of the normal levels. A single individual with one copy of the wild-type HD gene inactivated by chromosomal translocation is neurologically normal despite a reduction in HD expres-

sion level of about 50%,²⁵ and heterozygous knockout mice with an approximate 50% reduction of wild-type HD expression also display no abnormal phenotype.^{11,12} These observations suggest that a reduction in HD expression by 50% of normal level may be sufficient for neuronal survival in adults.

One potential pitfall of mRNA destruction with DNA enzymes is that the cleavage might occur in other mRNA species. For the following reasons, this is unlikely. First, it is known that HD sequence bears no similarity to other reported sequences with the exception of the CAG repeats, the adjacent CCG repeats, and a "Heat" motif.^{3,21,26} Neither of our optimal sites for enzyme cleavage (positions 9 and 421) are within those regions. Second, the length of the substrate-binding arms of our DNA enzymes are 16 bases for HD-9, and 21 bases for HD-421 (see Fig 3a). Such an interaction length should be unique in the human genome, and can be increased if necessary. Finally, the discrimination power of DNA enzymes may go well beyond the matched sequence, because local secondary structures must also allow access to the desired cleavage site. This is supported by the fact that 9 of our 15 DNA enzymes, although designed to match the HD mRNA perfectly, resulted in no cleavage at all.

In summary, we have demonstrated the first effective destruction of the HD mRNA with RNA-cleaving DNA enzymes. The cleavage of HD mRNA occurs in a sequence-specific manner, leads to significant reduc-

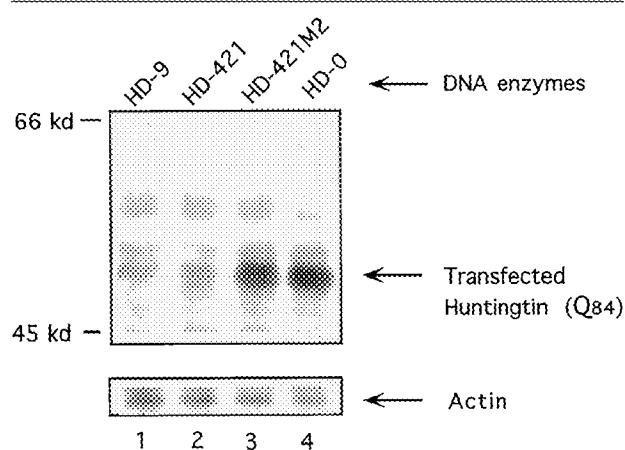


Fig 5. Western blot analysis of the effect of DNA enzymes on HD protein expression in cells. The expression plasmid containing a cytomegalovirus (CMV) promoter and HD exons 1 to 6 with 84 CAG repeats was cotransfected with the protected DNA enzymes into the HEK-293 cells. Lane 1, cells cotransfected with HD-9; lane 2, cells cotransfected with HD-421; lane 3, cells cotransfected with a control DNA enzyme identical to HD-421 except with no enzymatic activity because of a point mutation in its catalytic core (HD-421M2); lane 4, cells cotransfected with a DNA enzyme (HD-0) that produced no cleavage in vitro. Both HD-9 and HD-421 significantly reduced the level of transfected mutant HD protein in comparison with the controls. HD = Huntington's disease; HEK = human embryonic kidney (cells).

tion of HD protein expressed in mammalian cells, and the reduction of HD proteins is unlikely the result of an antisense effect. While there is currently no effective therapy for HD, decreasing the level of mutant huntingtin in cells with DNA enzymes could serve as a viable option. These DNA enzymes can also be used to study the function of HD gene in cells.

Although several antisense DNA drugs are now undergoing clinical trials,²⁷ a molecule such as a DNA enzyme that exhibits high specificity and catalytic activity may hold greater therapeutical potential. If a reliable delivery method for introducing DNA enzymes into cells were established, these molecules might provide effective therapy for HD and for similar gain-of-function, dominant genetic disorders.

This study was supported by the Hereditary Disease Foundation (L.Y.), and by Public Health Service grants NS29837 and NS33467 (R.G.K.).

We thank Dr Christopher Ross for providing the HD expression plasmids, and Dr Alan Sharp for polyclonal antibody AP-194. We also thank Dr Gerald Joyce and Dr Ronald Breaker for helpful suggestions on designing DNA enzymes, and Dr Robert Horvitz for critical suggestions on the manuscript.

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